Biotransformation of Hexahydro-1,3,5-trinitro-1,3,5-triazine Catalyzed by a NAD(P)H: Nitrate Oxidoreductase from *Aspergillus niger*

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Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) can be efficiently mineralized with anaerobic domestic sludge, but the initial enzymatic processes involved in its transformation are unknown. To test the hypothesis that the initial reaction involves reduction of nitro group(s), we designed experiments to test the ability of a nitrate reductase (EC 1.6.6.2) to catalyze the initial reaction leading to ring cleavage and subsequent decomposition. A nitrate reductase from *Aspergillus niger* catalyzed the biotransformation of RDX most effectively at pH 7.0 and 30 °C under anaerobic conditions using NADPH as electron donor. LC/MS (ES-) chromatograms showed the formation of hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) and methylenedinitramine as key initial products of RDX, but neither the dinitroso neither (DNX) nor trinitroso (TNX) derivatives were observed. None of the above detected products persisted, and their disappearance was accompanied by the accumulation of nitrous oxide (N_2O), formaldehyde (HCHO), and ammonium ion (NH_4^+). Stoichiometric studies showed that three NADPH molecules were consumed, and one molecule of methylenedinitramine was produced per RDX molecule. The carbon and nitrogen mass balances were 96.14% and 82.10%, respectively. The stoichiometries and mass balance measurements supported a mechanism involving initial transformation of RDX to MNX via a two-electron reduction mechanism. Subsequent reduction of MNX followed by rapid ring cleavage gave methylenedinitramine which in turn decomposed in water to produce quantitatively N_2O and HCHO. The results clearly indicate that an initial reduction of a nitro group by nitrate reductase is sufficient for the decomposition of RDX.

Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is widely used for military and commercial purposes due to its high explosive properties. The large scale manufacturing, use, and disposal of RDX has resulted in elevated levels of soil and groundwater contamination (*1*, *2*). RDX and its degradation products are toxic, mutagenic, and carcinogenic to humans and other biological systems (*3*, *4*). Hence there is an urgent need to remove it from the contaminated sites. Extensive literature is available for the biotransformation of RDX under anaerobic (*5*–*9*) and aerobic conditions (*10*–*13*), but little information is available regarding enzymatic degradation. Nitroreductases, largely from bacterial strains belonging to the Enterobacteriaceae family (*14*–*16*), are identified to play a role in the anaerobic bacterial transformation of RDX. So far little is known about the initial enzymatic processes involved in the transformation of RDX and its products.

RDX is a highly oxidized compound, and, therefore, a successful initial enzymatic attack on its nitro group(s) might destabilize the inner C–N bonds and lead to ring cleavage and spontaneous decomposition as has been demonstrated for its chemical decomposition (*17*). In our previous studies (*9*), we hypothesized that methylenedinitramine, a key RDX ring cleavage product in anaerobic sludge, may have been produced by three different routes, i.e. initial hydrolytic cleavage of a C–N bond, hydroxylation at C–H bond, and/ or via formation of hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX). However, the nature of enzyme(s) involved was not clear. Kitts et al. (*16*) have demonstrated the reduction of RDX by a type I nitroreductase from *Enterobacter cloacae,* but the mechanism and degradation products were not described. The present study is a continuation of our previous work and is carried out as a model system in order to understand the mechanism of initial enzymatic attack on RDX and its subsequent degradation as catalyzed by a single purified nitroreductase.

We selected a nitrate reductase (EC 1.6.6.2) from a fungus *Aspergillus niger* to transform RDX under anaerobic conditions because nitrate reductases are ubiquitous enzymes in diverse groups of microorganisms, especially denitrifying bacteria, and their physiological role is to reduce nitrate to nitrite via a two-electron transfer (*18*–*21*). Hence there is a great possibility that either this or similar enzyme may be one of the key enzymes responsible for RDX transformation in anaerobic sludge. So far, no information is available regarding nitrate reductase reaction with cyclic nitramine compounds such as RDX. In the present study, we employed this enzyme to degrade RDX and to gain a new insight into the initial enzymatic processes involved in the transformation of this energetic chemical.

Experimental Section

Chemicals. Commercial grade RDX (purity > 99%) was provided by Defence Research Establishment, Valcartier, Quebec, Canada (*6*). MNX (95% purity) was synthesized according to the method described by Brockman et al. (*22*). For the experiment describing MNX biotransformation with nitrate reductase the chemical (>99% purity) was obtained from SRI International (Menlo Park, CA). *Aspergillus niger* NAD(P)H: nitrate oxidoreductase (EC 1.6.6.2), NADPH, and formaldehyde were obtained from Sigma chemicals, Canada. Methylenedinitramine was obtained from the rare chemical department of Aldrich, Oakville, ON, Canada. Hydrazine and formamide were purchased from Aldrich, Canada. Standard nitrous oxide (N_2O, 980 ppm by mole) was obtained from Scott specialty gases, Sarnia, ON, Canada.

Enzyme Preparation and Assays. Lyophilized enzyme was suspended in potassium phosphate buffer (50 mM) at pH 7.0 and washed thrice with 2.5 mL of buffer using Biomax-

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5K membrane centrifuge filter units (Sigma chemicals). The washed enzyme was then suspended in 0.5 mL of buffer, and the protein concentration was measured by bicinchoninic acid (BCA) kit (Sigma chemicals) using bovine serum albumin as standard. The native enzyme activity was estimated spectrophotometrically at 340 nm as the rate of oxidation of NADPH in the presence of nitrate.

Nitrate reductase catalyzed RDX transformation was performed under anaerobic conditions in 6 mL glass vials containing 1 mL of reaction mixture sealed under an atmosphere of argon. The above assay mixture contained RDX (or MNX) (100 μM), NADPH (300 μM), and 1 mg of enzyme (equivalent to 0.25 native units) in potassium phosphate buffer (50 mM) at pH 7.0 and 30 °C. Three different controls were prepared as follows: (1) RDX and NADPH in buffer (i.e. without enzyme); (2) RDX and enzyme in buffer (i.e. in the absence of NADPH); and (3) RDX only in buffer. The samples from liquid and gas phases were withdrawn periodically to analyze for RDX and transformation products as described below. The enzymatic transformation rate [V] of RDX was expressed as μmol of RDX transformed h^{-1} mg^{-1} protein.

To study the fate of formamide and hydrazine in the reaction mixture, each chemical (50 μM) was incubated separately with nitrate reductase and NADPH for 1 h at pH 7.0 and 30 °C under the same conditions as described above. The residual compounds and their products were analyzed as stated below.

Analytical Procedures. RDX was analyzed by a reversed phase-high-pressure liquid chromatograph (RP-HPLC) connected to photodiode array (PDA) detector ($\lambda_{254\ nm}$). Samples (50 μL) were injected into a Supelcosil LC-CN column (4.6 mm ID × 25 cm) (Supelco, Bellafonte, PA), and the analytes were eluted using methanol/water gradient at a flow rate of 1.5 mL/min (*12*). The MNX and methylenedinitramine were analyzed by a Micromass benchtop single quadrupole mass detector attached to a Hewlett-Packard 1100 series HPLC system equipped with a photodiode array detector (*9*). Ionization was carried out in a negative electrospray ionization mode ES(−) giving mainly deprotonated molecular mass ion [M − H]. Their identities were also confirmed by comparison with reference standards.

Formaldehyde (HCHO), nitrous oxide (N_2O), and ammonium (NH_4^+) were analyzed as described in our previous studies (*13*).

Hydrazine was analyzed by a HPLC system equipped with a Waters model 600 pump (Waters Associates, Milford, MA), a 717 plus autosampler, a Hamilton RPX-X200 analytical cation exchange column (250 mm × 4.1 mm), a Waters postcolumn reaction module with a Waters reagent manager pump, a Waters model 464 electrochemical detector with a gold-working electrode, and a base resistant Ag/AgCl reference electrode. The eluent was 6% v/v acetonitrile in 0.005 M KH_2PO_4 solution in deionized water. The eluent was degassed by continuous helium sparging before and during use. The postcolumn reaction solution was 0.1 M NaOH solution in deionized water. The operating parameters for the system were: eluent flow rate, 1.0 mL/min; temperature, 30 °C; injection volume, 25 μL; flow-rate of postcolumn reaction solution, 250 μL/min; working electrode cleaning potential, 500 mV (0.333 s); pretreatment potential, −350 mV (0.333 s) and measuring potential, 100 mV in DC mode.

Formamide was analyzed by a Micromass benchtop single quadrupole mass detector attached to an HPLC system equipped with a photodiode array detector and a synergi polar-RP column (4.6 mm ID × 15 cm) (Phenomenex, Torrance, CA) at 25 °C. The solvent system was a methanol/water gradient (10−90% v/v) at a flow rate of 0.75 mL/min. For mass analysis, the ionization was carried out in a positive electrospray ionization mode ES(+) producing mainly the
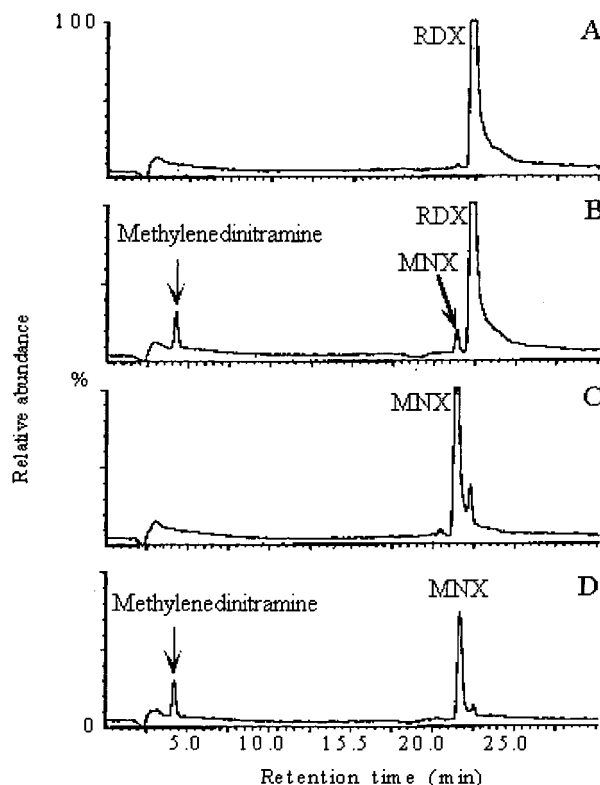

FIGURE 1. LC/MS (ES-) chromatogram of RDX and MNX transformation by nitrate reductase at pH 7. A and C: RDX and MNX, respectively, at 0 min. B and D: RDX and MNX, respectively, after 60 min showing the formation of methylene dinitramine.

protonated molecular mass ion [M + H]. The electrospray probe tip potential was set at 3.5 kV with a cone voltage of 35 V at an ion source temperature of 150 °C.

Results and Discussion

Biotransformation Conditions and Product Identification. We found that *A. niger* nitrate reductase catalyzed the transformation of RDX in the presence of NADPH as electron donor. The transformation was optimal under anaerobic conditions at pH 7.0 and 30 °C (data not shown). The disappearance of RDX was accompanied by the formation of MNX and the ring cleavage product methylenedinitramine (Figure 1 A&B). In control experiments, no degradation was observed except in control number 1 which contained NADPH and RDX (see Experimental Section) where a negligible RDX loss (<3% of total RDX degradation) was observed in 1 h of reaction time.

To strengthen our hypothesis that RDX is first reduced to MNX and the latter undergoes further reduction to finally produce methylene dinitramine, $(O_2NNH)_2CH_2$, we incubated the standard MNX with nitrate reductase under similar reaction conditions as those of RDX biotransformation (see Experimental Section). As a result, we observed the formation of $(O_2NNH)_2CH_2$ from MNX (Figure 1C,D). Figure 1 is a typical LC/MS (ES−) chromatogram obtained after incubating RDX and MNX in separate vials with nitrate reductase for 60 min under the optimal reaction conditions. Methylenedinitramine and MNX were identified by comparing their chromatographic retention times and LC/MS (ES−) spectra with those of reference standards. MNX exhibited a retention time of 20.25 min and produced characteristic mass fragments at 46 and 251 Da, representing NO_2 and an unidentified adduct (205 Da from [M − H] of MNX and 46 Da), respectively. We were unable to detect the [M − H] for MNX. Whereas, methylenedinitramine exhibited a characteristic mass data
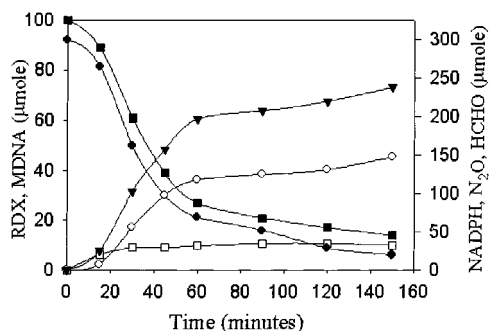
FIGURE 2. A time course of nitrate reductase catalyzed RDX transformation: ■ RDX; □ methylenedinitramine ○ N$_2$O; ● NADPH; ▼ HCHO. Relative standard deviations were within 7%.

TABLE 1. Carbon and Nitrogen Mass Balance and Stoichiometry of Metabolites Produced during RDX Transformation Catalyzed by Nitrate Reductase from *Aspergillus niger* after 150 min of Reaction

reactants/metabolites	concn of reactants/ metabolites (μmol)	% carbon recovery[a]	% nitrogen recovery[a]
1. RDX	86	100	100
2. NADPH	280	NA	NA
3. methylenedinitramine (MDNA)	10	3.87	7.75
4. formaldehyde (HCHO)	238	92.27	NA
5. nitrous oxide (N$_2$O)	148	NA	57.37
6. ammonium ion (NH$_4^+$)	88	NA	16.98
total % mass recovery		96.14	82.10

[a] Calculated from the total carbon and nitrogen mass in the transformed RDX (86 μmol); NA, not applicable. Standard deviations were within 7% of the mean absolute value ($n = 3$).

at 61 and 135 Da, representing NHNO$_2$ group and the [M − H] mass ion of the parent compound as we have reported previously (*9*). A significant observation in Figure 1 is the absence of the dinitroso (DNX) and trinitroso (TNX) products of RDX.

None of the products in Figure 1 persisted, and their disappearance was accompanied by the accumulation of formaldehyde (HCHO), ammonium ion (NH$_4^+$), and nitrous oxide (N$_2$O). The product distribution was similar to that obtained during biodegradation of RDX with domestic anaerobic sludge (*9*). The main difference between the two systems is the subsequent mineralization of HCHO in the presence of sludge (*9*). The latter is expected to contain a complex consortium of microbes with enzyme(s) for the biodegradation of HCHO.

Stoichiometry and Pathway. In the time course study, methylenedinitramine was observed as the prime intermediate, and N$_2$O and HCHO were the major end products (Figure 2). Throughout the reaction course the concentration of methylenedinitramine was low and did not exceed 10 μmol because it is quite unstable at pH 7.0 and 30 °C and rapidly decomposed into N$_2$O and HCHO. The time-course and stoichiometry of abiotic decomposition of methylenedinitramine in water has been reported in one of our previous works (*9*). The phenomenon of quantitative decomposition of methylenedinitramine into two molecules of N$_2$O and one molecule of HCHO was used in the present study to calculate the stoichiometry of RDX biotransformation with the enzyme. Table 1 shows that 86 μmol of RDX produced 148 μmol of N$_2$O and 10 μmol of methylenedinitramine (equivalent to 20 μmol of N$_2$O). Hence we concluded that for each reacted molecule of RDX only one molecule of methylenedinitramine, containing two N−NO$_2$ groups, was produced.

A part of the remaining N−NO$_2$ group in RDX apparently leads to the formation of ammonium ion (88 μmol). The total nitrogen mass balance was 82.10% and was distributed between N$_2$O (57.37%), methylenedinitramine (7.75%), and ammonium ion (16.98%) (Table 1). The carbon mass balance was 96.13% and was distributed between HCHO (92.27%) and methylenedinitramine (3.87%) which indicated that all the recovered carbon was in the form of HCHO. As a result, it was concluded that one RDX molecule produced three HCHO molecules (Figure 3).

It was difficult to quantify MNX since it existed only in trace amounts throughout the RDX reaction course. It was assumed that only MNX (with two N−NO$_2$) can produce methylenedinitramine (with two N−NO$_2$), and neither DNX (with only one N−NO$_2$) nor TNX (with no N−NO$_2$) can act as a precursor to methylenedinitramine. In addition as we described previously none of the two compounds was detected. Finally we found that for each reacted RDX molecule, roughly three molecules of NADPH, representing six electrons, were consumed (Table 1).

Based on the above stoichiometry, we proposed a degradation pathway for RDX as shown in Figure 3. RDX was presumed to undergo reduction in three sequential steps to first produce MNX, followed by the formation of the hydroxylamine derivative RDX-NHOH (I), which then reduced further to the amine metabolite RDX-NH$_2$ (II). None of the two hypothetical compounds (I and II) was detected. If formed both I and II are expected to be unstable in water and might undergo rapid ring cleavage to produce an unstable hydroxyalkylnitramine product (III) (Figure 3). Alkylhydroxylamines are reportedly unstable in water (*23*), and thus III should undergo rapid decomposition to produce methylenedinitramine, HCHO, and another hypothetical compound possibly methanolhydrazine (VI) (NH$_2$NHCH$_2$OH). The latter being a form of α-hydroxylamine should also decompose in water since we could not detect it. The decomposition of VI was presumed to produce HCHO and NH$_4^+$ possibly by two routes, i.e. via formation of formamide and/or hydrazine (Figure 3). However, we did not detect either of the two compounds probably because they did not accumulate. For instance, when either formamide (50 μM) or hydrazine (50 μM) was incubated with nitrate reductase under similar reaction conditions to those described for RDX, roughly 50% of the formamide was converted to HCHO and NH$_4^+$ and 40% of the hydrazine to NH$_4^+$. The unaccounted starting materials could not be traced probably because of their reactivity with reaction mixture components such as protein, buffer, and/or NADPH.

We were able to account for 82.10% of the total nitrogen mass content of RDX; we speculated that the missing 18% nitrogen mass existed in form of an unidentified compound that might have reacted with other components as mentioned above. In particular, hydrazine is a very reactive compound, and it is known to polymerize with HCHO (*24, 25*). Hydrazine formation was first proposed by McCormick et al. (*5*) during RDX degradation by an anaerobic sludge, and since then there has been no other report that describes its formation from the biodegradation of cyclic nitramine explosives. We excluded DNX and TNX from the RDX biotransformation pathway (Figure 3) based on our observation that none could be detected by LC/MS (ES−). Furthermore, neither of the two nitroso products can produce methylenedinitramine. In contrast, the stoichiometric formation of methylenedinitramine (one μmole of RDX produced 1 μmol of methylenedinitramine) confirmed that ring cleavage proceeded via MNX (Figure 3). In support of this when we incubated MNX with nitrate reductase under the same conditions used previously with RDX we observed the formation of methylene dinitramine (Figures 1C,D) as a key ring cleavage product which also decomposed to HCHO and N$_2$O. Once again neither DNX nor TNX was observed during MNX incubation with nitrate reductase. However, DNX and TNX have been

FIGURE 3. A postulated transformation pathway of RDX as initiated by nitrate reductase. Small numbers above carbon atoms demonstrate location of bond cleavage.

reportedly observed during RDX degradation by a consortium containing *Serratia marcescence* (*15*), by anaerobic sludge (*5, 7, 8*) and by soil slurries (*26−28*) which further indicate the possibility of some other mechanism(s) operating in the complex environments.

Role of Nitrate Reductase in the Initial Transformation of RDX. Eukaryotic nitrate reductases (EC 1.6.6.1-3) contain three redox cofactors, i.e. FAD, heme-Fe, and molybdopterin (Mo-MPT). FAD is the oxidation site for NAD(P)H and the subsequent electron-transfer proceeds through a heme-Fe center to the molybdopterin which is a reduction site for nitrate (*21*). It is known that this enzyme reduces nitrate to nitrite via a two-electron-transfer mechanism, and this reaction takes place at the Mo-MPT cofactor site (*19−21*). During nitrate reduction, the molybdenum (Mo) present in the Mo-MPT cofactor frequently changes its oxidation state from oxidized (Mo^{VI}) to reduced (Mo^{IV}) and *vice-versa* by receiving two electrons from the heme-Fe center and transferring them to nitrate. In the present study, nitrate appeared to be a competitive inhibitor of RDX biotransformation as evident by the Lineweaver−Burk's plots in the presence of increasing concentration of nitrate in the reaction mixture (Figure 4). This indicated that nitrate and RDX might be sharing a common binding site on the enzyme (i.e. Mo-MPT cofactor).

We found that 86 μmol of RDX transformation was accompanied by the consumption of 280 μmol of NADPH, indicating that roughly three NADPH molecules were consumed during transformation of one RDX molecule under anaerobic conditions (Table 1). Therefore, we presume that a total of six electrons (or three NADPH) participated in the complete transformation of RDX. Since RDX requires an obligatory two electrons to be transformed to MNX, it is concluded that nitrate reductase might have catalyzed two-electron transfer to the RDX in the first reduction step to produce MNX. Thereafter, the remaining two pairs of electrons were presumed to get involved in the sequential reduction of MNX to RDX-NHOH (I) (1 NADPH) and RDX-NH2 (II) (1 NADPH) (Figure 3). However, we cannot rule out the possibility that RDX-NHOH, being unstable, might also undergo ring cleavage. Under these circumstances, the last pair of electrons (third NADPH molecule) would be utilized after the ring cleavage.

FIGURE 4. Lineweaver-Burk's plots showing nitrate as the competitive inhibitor of nitrate reductase catalyzed RDX transformation: (●) 0; (○) 20 μM; (▼)100 μM nitrate.

As far as we are aware, there are no reports that describe the transformation of cyclic nitramine compound(s) with nitrate reductase(s). However, the enzyme-catalyzed two-electron reduction of nitroaromatic compounds to nitroso derivatives by NAD(P)H:quinone reductase (*29, 30*) and bacterial nitroreductases (*31−35*) have been reported. Peterson et al. (*31*) demonstrated the use of type I (a two-electron-transfer process) oxygen insensitive nitroreductase from *Escherichia coli* to catalyze reduction of nitro groups (*31*). Whereas Kitts et al. (*16*) demonstrated the reduction of RDX and TNT by a type I oxygen insensitive nitroreductase from *Enterobacter cloacae* without elaborating on the mechanism or degradation products. It was earlier reported that the prime function of nitroreductases is to reduce only nitro groups of nitroaromatic compounds, and they cannot reduce inorganic *N*-oxides nitrate or nitrite (*32, 36*). On the other hand, Averill (*37*) reported that nitrate is chemically analogous to the organic nitro group (Ar−NO2) which indirectly indicates that nitrate reductase may function as nitroreductase. In the present study, nitrate reductase from *A. niger* seemed to share the functional similarity (Figure 3) with type I nitroreductase because the latter also catalyzes a two-electron-transfer process (*31−34*).

The results discussed above have clearly demonstrated that MNX is the first reduced product of RDX formed by a two-electron reduction catalyzed by nitrate reductase. MNX underwent further reduction followed by the ring cleavage and subsequent decomposition to produce HCHO, NH_4^+, N_2O. This study strongly supported our previous hypothesis (9) of the involvement of MNX as an initial RDX metabolite prior to ring cleavage. However, it remains to be explored whether nitrate reductase or similar enzymes are present in the microorganisms inhabiting the anaerobic sludge.

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